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Discovery of Allelic Variants of *HOXA1* and *HOXB1*: Genetic Susceptibility to Autism Spectrum Disorders

JENNIFER L. INGRAM,[1,2] CHRISTOPHER J. STODGELL,[1] SUSAN L. HYMAN,[3] DENISE A. FIGLEWICZ,[4] LOWELL R. WEITKAMP,[5] AND PATRICIA M. RODIER[1]*

[1]*Department of Obstetrics and Gynecology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642*
[2]*Department of Environmental Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642*
[3]*Department of Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642*
[4]*Department of Neurology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642*
[5]*Department of Psychiatry, Division of Genetics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642*

ABSTRACT

Background: Family studies have demonstrated that the autism spectrum disorders (ASDs) have a major genetic etiologic component, but expression and penetrance of the phenotype are variable. Mice with null mutations of *Hoxa1* or *Hoxb1*, two genes critical to hindbrain development, have phenotypic features frequently observed in autism, but no naturally occurring variants of either gene have been identified in mammals.

Methods: By sequencing regions of genomic DNA of patients with autism spectrum disorders, we detected a substitution variant at *HOXA1* and an insertion variant at *HOXB1*, both in coding regions of the genes. Fifty-seven individuals ascertained for a diagnosis of an ASD, along with 166 of their relatives, were typed for these variants. Two non-ASD populations were typed, and the frequency of the newly identified alleles was determined in all groups. The genotypes of the ASD families were tested for conformation to Hardy-Weinberg proportions and Mendelian expectations for gene transmission.

Results: The frequency of the variants was 10–25% in persons of European or African origin. In the ASD families, there was a significant deviation from the *HOXA1* genotype ratios expected from Hardy-Weinberg proportions ($P = 0.005$). Among affected offspring, a significant deviation from Mendelian expectation in gene transmission ($P = 0.011$) was observed. No statistically significant effects were detected when the same analyses were applied to the *HOXB1* locus, but there was evidence of an interaction between *HOXA1*, *HOXB1*, and gender in susceptibility to ASDs.

Conclusions: The results support a role for *HOXA1* in susceptibility to autism, and add to the existing body of evidence implicating early brain stem injury in the etiology of ASDs.

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INTRODUCTION

The symptoms of autism spectrum disorders—autism, Asperger syndrome, childhood disintegrative disorder, and pervasive developmental disorder-not otherwise specified—include deficits in social interaction and communication as well as restricted and repetitive patterns of interests and activities. The prevalence rate of this spectrum of disabilities is greater than 1/1,000 births and may be as high as 6/1,000 (Bryson et al., '88; Bryson and Smith, '98), making it one of the most common congenital disorders. The causes are widely acknowledged to be multiple. Family studies of the pattern of inheritance support a genetic etiology. The rate of concordance for the diagnosis of autism in monozygotic twins is at least 60% (Folstein and Rutter,

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Jennifer L. Ingram and Christopher J. Stodgell contributed equally to this paper.

*Correspondence to: Patricia M. Rodier, Department of Obstetrics and Gynecology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642.
E-mail: patricia_rodier@urmc.rochester.edu

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'77; Bailey et al., '95). The concordance rate for the disorder in dizygotic twins and siblings is about 3–6% (Smalley et al., '88). Even more striking are the concordance rates for symptoms short of the full diagnosis among relatives of individuals with autism. Concordance for some symptoms is 86% for monozygotic twins and 15% for dizygotic twins or siblings (Folstein and Rutter, '77; Bailey et al., '95). Language symptoms are significantly more common in parents of children with autism spectrum disorders (ASDs) than in parents of children with other disabilities (Landa et al., '91, '92), and social difficulties are reported frequently in both first- and second-degree relatives (Volkmar et al., '98). Analyzing the distribution of the "lesser variant" of autism in families with ASDs, Szatmari and colleagues demonstrated that it is a variable phenotype of symptoms of the disorder that clusters in families, rather than a specific diagnosis (MacLean et al., '99).

The finding of a high rate of autism in individuals exposed to thalidomide early in gestation indicates that the disorder can be induced by teratogens (Strömland et al., '94). Valproic acid has been implicated as a second teratogen that increases the risk of autism (Christianson et al., '94; Williams and Hersh, '97) and ethanol as a third (Nanson, '92; Harris et al., '95; Aronson et al., '97). The teratologic findings are especially important, for they indicate that autism, although diagnosed on the basis of behavioral symptoms alone, is sometimes accompanied by very specific neurologic and morphologic symptoms. The thalidomide cases exhibited deficits of function of the oculomotor, abducens, and facial cranial nerves. Each case had hearing deficits and malformed ears. All these features have long been known to occur after in utero exposure to thalidomide (d'Avignon and Barr, '64). Elevated frequencies of anomalies of cranial nerve function (Rosenhall et al., '88; Scharré and Creedon, '92), hearing deficits (Klin, '93), and ear malformations (Walker, '77; Rodier et al., '97), have been reported in idiopathic cases of autism, as well, but have been considered to be of minor importance, compared with the overwhelming behavioral symptoms. However, the nonbehavioral symptoms, whether they represent major disabilities or not, may be of significant value in understanding the etiology of the autism spectrum, because they indicate the embryological stage at which the disorder arises.

The results of thalidomide exposure have been studied in thousands of cases, and the stages of embryogenesis at which specific morphologic outcomes are induced by the drug are well known (Miller, '91). Thus, the critical period when thalidomide exposure leads to autism can be deduced from the external anomalies of the patients. Of 86 cases evaluated, only 15 had ear malformations without the limb malformations that result from later injury (Miller, '91; Strömland et al., '94). Four of the 15 cases had autism. Another case of autism had a combination of ear and limb defects, probably representing repeated injury, but there were no cases of autism among the patients with limb malformations and normal ears. The results indicate that all the patients with autism were injured between the 20th and the 24th days after conception. This is the time when the neural tube is forming and the central nervous system (CNS) is divided into rhombomeres.

Very few neurons form as early as this phase of CNS development. Most are motor neurons for the cranial nerves—those that control the movement of the eyes, face, tongue, jaw, throat, and larynx (Bayer et al., '93). The dysfunction of some of the same innervation in thalidomide-induced autism and idiopathic cases provides support for the critical period defined from ear effects, as does the presence of ear malformations in children with valproate-induced autism (Christianson et al., '94; Williams and Hersh, '97). Both ear anomalies (Binkerd et al., '88) and reductions in the number of neurons in several cranial nerve nuclei (Rodier et al., '96) have been reported in animals exposed experimentally to valproic acid during early gestation. A histological study of the brain in a well-documented case of idiopathic autism showed near-absence of both the facial nucleus and the superior olive, an auditory relay nucleus (Rodier et al., '96). The brain was shortened at the pontomedullary junction, indicating that the missing structures had failed to form, rather than being lost at a later stage of development.

The pattern of deficits in the human brain suggests a failure of development of at least one of the rhombomeres, the fifth, from which part of the facial nucleus and all of the superior olive are thought to arise (e.g., Lumsden and Keynes, '89; Marin and Puelles, '95). Null mutations of early developmental genes create similar morphologic deficits in mice. For example, mouse knockouts of *Hoxa1* lack the superior olive, the abducens nucleus, and all but the most anterior part of the facial nucleus, suggesting deficient development of the 4th, 5th, and 6th rhombomeres (Carpenter et al., '93; Mark et al., '93). The animals lacking *Hoxa1* function also have malformations of the inner, middle, and external ears (Carpenter et al., '93). The anatomy of the *Hoxb1* knockout mouse has not been described in the same detail but displays an even greater deficit of facial nucleus neurons and the expected neurologic dysfunction in the muscles of facial expression (Goddard et al., '96; Studer et al., '96). *Hoxa1* and *Hoxb1* are paralogous genes that have evolved from a single ancestral source analogous to the *labial* gene of *Drosophila* (Ruddle et al., '94). They now appear on different chromosomes — 6 and 11 in the mouse, and 7p and 17q in the human. Both are expressed in the hindbrain exclusively during the period of neural tube formation (Murphy and Hill, '91), the same period when the thalidomide cases were injured. The two genes have maintained similarities of both sequence and function and have been shown to act synergistically in determining segmentation and identity in the developing hindbrain and associated structures (Gavalas et al., '98).

No naturally occurring variants of *Hoxa1* or *Hoxb1* have been detected in any mammalian species. However, variants of other *Hox* genes critical to the development of more caudal structures have been shown to cause severe human birth defects. A mutation of *HOXD13* is a cause of synpolydactyly (Muragaki et al.,

'96), and a mutation of *HOXA13* is a cause of hand-foot-genital syndrome (Mortlock and Innis, '97). Thus, the *HOXA1* and *HOXB1* loci are candidates for markers of genetic susceptibility to the autism spectrum disorders. We report polymorphisms for both *HOXA1* and *HOXB1* in humans; analyses suggesting that these loci, especially *HOXA1*, may play a role in susceptibility to autism.

SUBJECTS AND METHODS

Subjects with autism and their relatives

The subject populations included 57 probands, 46 male and 11 female, and 166 relatives of these probands. Characteristics of the probands are summarized in Table 1. Forty-nine of the probands were ascertained through records of the Strong Center for Developmental Disabilities in Rochester, New York. The other eight probands were identified by the same methods at various university medical centers in the United States. All 57 probands were from Caucasian backgrounds.

All probands met DSM-IV (American Psychiatric Association, '94) criteria for autism, Asperger syndrome, pervasive developmental disorder (not otherwise specified), or childhood disintegrative disorder. All had been rated on the Childhood Autism Rating Scale (Schopler et al., '80) or on the Autism Diagnostic Inventory - Revised and Autism Diagnostic Observation Schedule (Lord et al., '89; Lord et al., '94). In every case, the diagnosis had been confirmed by at least two experienced clinicians.

Fifty-two of the probands had a parental report of a first-, second-, or third-degree relative with a history of one of the autism spectrum disorders or a first-degree relative who was brought to medical attention for language delay. Language delay occurs in families with no cases of autism, but it frequently occurs in first-degree relatives of people with ASDs and may therefore share a common genetic etiology. Since language delay typically results in medical attention, families can report this symptom without imposing their own judgment, as they would for social disability, obsessive interests, or many other symptoms of the autism spectrum. Thirty-two of the probands had physical features related to the functions of the candidate genes (e.g., esotropia, ear malformations), most of which were minor. This proportion is similar to that reported in assessments of minor malformations in autism (e.g., Steg and Rapoport, '75). Among the probands, 72% had mental retardation and 81% were male. Both fractions are similar to the data reported for autism spectrum disorders in unselected samples (Bryson et al., '88). None of the probands had phenylketonuria or tuberous sclerosis, genetic disorders previously associated with autism, or fragile X syndrome.

Among the 166 relatives of probands from whom we acquired DNA samples, 32 persons met our criteria for being affected (10 with language delay and 22 with one of the autism spectrum disorders). Twenty-three of these (14 male and 9 female) were first-degree relatives of probands, five (all male) were second-degree relatives, three (two male and one female) were third-degree relatives, and one (male) was genetically unrelated. Thus, 68 of the 89 affected individuals were male, consistent with the established higher prevalence of ASDs in males. Two probands with autism, 083B and 086D, had monozygotic twins (One twin had PDD and the other had a minor language delay. Both were counted as affected). For purposes of analysis, each twin pair was considered a single genetic event. Of the 134 relatives who did not meet the criteria for being affected, 121 (56 male and 65 female) had a first-degree relationship to a proband, and 13 (five male and eight female) had a second-degree relationship to a proband.

Forty-seven probands had both parents available for the study. In addition, two of the families just described provided three additional sets of parent-affected offspring nuclear families for analysis. In the 50 nuclear families in which both parents were typed for *HOXA1* and *HOXB1* there were 66 affected offspring (59 who met DSM-IV criteria for autism spectrum disorders and 7 who had a language delay) and 16 typed offspring who were unaffected. Blood samples were not taken from two affected and 24 unaffected offspring in these 50 nuclear families.

Other subjects

Two groups without ASD diagnoses were examined for the frequency of *HOXA1*and *HOXB1* variants. The first consisted of a convenience population of 119 unrelated adults recruited from five different medical centers in North America. All were spouses or other unrelated family members of patients with late onset neurological disorders. The sex and ethnic composition of this group are unknown, but most were probably Caucasian. The second group consisted of ten individuals from each of nine different populations in the Coriel Human Diversity Panel (Table 2).

Detection of polymorphic alleles of *HOXA1* and *HOXB1*

After written consent was obtained, and with the approval of the University's Institutional Review Board, blood was collected from probands with an autism spectrum disorder and all available family members. DNA was extracted from the blood using phenol and chloroform, after isolation and lysis of the white blood cells. DNA extraction from fixed brain tissue was accomplished with Qiagen's QiaAmp tissue kit, following the manufacturer's instructions.

The first exons of human *HOXA1* and *HOXB1* were polymerase chain reaction (PCR)-amplified with the following oligonucleotide primers: *HOXA1*, (sense) 5′-GCAAGAATGAACTCCTTCCTG-3′, (antisense) 5′-ACCAACCAGCAGGACTGACCT-3′; *HOXB1*, (sense) 5′-GCATGGACTATAATAGGATG-3′, (antisense) 5′-TCTTGGGTGGGTTTCTCTTA-3′. PCR was carried out using Ready-To-Go PCR Beads (Pharmacia Biotech) according to the manufacturer's instructions. Amplification for *HOXA1* consisted of 35 cycles of 94°C (for 45 sec); 62°C (for 45 sec); 72°C (for 35 sec), producing a

TABLE 1. Characteristics of 57 probands with autism spectrum disorders

ID #	Genotype	Gender	Familial	IQ	Diagnosis	Physical, neurological features
001C	A/A, +/+	M	Y		Autism	Brachydactyly type 4
003D	A/G, +/+	M	Y		Asperger	Simple ears with detached lobes
004A	A/A, +/INS	F	N	MR	Autism	High palate, cranial nerve palsies
005A	A/G, +/+	F	N	MR	Autism	Broad nasal root, hypertelorism, esotropia, middle ear dysplasia
006A	A/A, +/+	M	Y	MR	Autism	Flat nasal bridge
007A	A/A/, +/INS	M	Y		Asperger	Ears—folded external helix
008B	A/A, +/INS	M	Y		Asperger	
009A	A/A, +/INS	M	Y	MR	Autism	Ears—prominent
011A	A/G, +/+	M	Y		Autism	Asymmetric ears, simple pinna
013A	A/A, +/+	M	Y	MR	Autism	Ears—posteriorly rotated, cheek hemangioma
014A	A/A, +/+	M	Y	MR	PDD	
015E3	A/G, +/INS	F	Y		Asperger	
016A	A/A, +/+	M	Y	MR	Autism	Ears—folded external helix
017A	A/G. +/+	M	Y		Asperger	
018A	A/G/ +/+	M	Y	MR	PDD	
019A	A/A, +/INS	M	Y	MR	Autism	Exotropia, unequal pupils
020A	A/A, +/+	M	Y	MR	Autism	Hypertelorism, cup-shaped ears, clinodactyly of fifth digit
021A	A/A, +/INS	M	Y		Asperger	Simple ears, brachydactyly type 4
023A	A/G, +/+	F	Y	MR	Autism	Ears—flattened superior helix
024A	G/G., +/INS	M	N	MR	Autism	Hypertelorism, downturned outer canthus, high palate
025C	A/A, +/INS	M	Y	MR	Autism	
026C	A/A, +/INS	M	Y	MR	Disintegrative disorder	
027A	A/A. +/INS	M	Y	MR	Autism	
029C	A/G, +/+	M	Y	MR	Autism	
030C	A/A, +/+	M	Y		Asperger	Tourette syndrome
031C	A/G, +/+	M	Y	MR	Autism	
032C	A/A, +/+	M	Y	MR	Autism	Unilateral anotia, Möbius syndrome, limb defects, scoliosis
034C	A/A, +/INS	M	Y	MR	Autism	Prominent ears
035B	A/A, +/INS	M	Y	MR	Autism	Hypertelorism, narrowed DIP joints
036C	A/A, +/INS	F	Y	MR	PDD	Clinodactyly of fifth digit
037C	A/G, +/INS	M	Y	MR	Autism	Hypertelorism
038C	A/G, +/+	M	Y		Asperger	
039C	A/G, +/+	M	Y	MR	Autism	Esotropia
040C	A/A, +/INS	M	Y	MR	Autism	Asymmetric ears, folded external helix, clinodactyly of fifth digit
041C	A/A, +/INS	F	Y	MR	PDD	
043C	A/G, +/+	M	Y	MR	Autism	
045B	A/A, INS/INS	M	Y	MR	Autism	Strabismus
046C	A/A, +/INS	F	Y	MR	Autism	Simple ears, preauricular pit, strabismus, high palate
047B	A/A, +/INS	M	Y		Asperger	
048C	A/A, +/INS	M	Y	High PIQ Low VIQ	Autism	Simple right ear, strabismus
049C	A/A, +/+	M	Y	MR	Autism	Head circumference = 5th percentile
050B	A/A, +/INS	M	Y	MR	Autism	
051C	A/G, +/+	F	Y	MR	Autism	Simple ears
052C	A/A, INS/INS	M	Y	MR	Autism	Telecanthus, congenital alopecia over occiput
054C	A/G, +/+	M	Y	MR	Autism	
055C	A/A, +/+	M	Y	MR	Autism	
057C	A/A, +/INS	M	Y		Asperger	Prominent ears
060C	A/A, +/+	M	Y	MR	Autism	
061C	A/A, +/+	F	Y	MR	Autism	
063A	A/G, +/+	M	Adopted	MR	PDD	Fetal alcohol syndrome (FAS)
066B	A/G, +/INS	M	Y	MR	Autism	Marfan syndrome, simple ears
76C	A/A, +/+	F	Y	MR	PDD	One ear cup-shaped
79C	A/A, +/+	M	Y		Autism	
081A	A/G, +/+	M	N	MR	Autism	Facial dyplegia, hypertelorism, ptossis, blind
083B	A/G, +/INS	M	Y	MR	Autism	
084D	A/G, +/INS	M	Y	MR	Autism	Syndactylly
086D	A/G, +/INS	F	Y	MR	Autism	

PDD, Pervasive Developmental Disorder; MR, Mental Retardation.

TABLE 2. Frequency of *HOXA1* and *HOXB1* genotypes in various populations

	Genotypes					
	HOXA1			*HOXB1*		
	A/A	A/G	G/G	+/+	+/INS	INS/INS
Convenience population	93	26	0	72	42	5
Coriel diversity panel						
Northern European	5	5	0	5	5	0
Middle Eastern	7	3	0	6	3	1
Indian/Pakistani	8	2	0	9	1	0
Mexican	5	5	0	9	1	0
Puerto Rican	6	4	0	8	2	0
African American	4	6	0	6	3	1
Southwest American Indian	10	0	0	10	0	0
Japanese	10	0	0	9	1	0
Chinese	10	0	0	10	0	0
ASD probands	35	21	1	30	25	2
Relatives of ASD probands						
Affected—ASD	14	8	0	9	10	2
Affected—language delay	5	5	0	6	3	1
Unaffected	90	44	0	77	54	3

ASD, autism spectrum disorder.

661-bp fragment. Amplification for *HOXB1* consisted of 35 cycles of 94°C (for 45 sec); 57°C (for 45 sec); 72°C (for 45 sec), resulting in a 576-bp fragment. The amplicons were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

Amplicons were sequenced with the ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences) according to the manufacturer's instructions. Sequencing reactions were electrophoresed on a 6% polyacrylamide sequencing gel. Each product was sequenced in both directions.

Nucleic acid sequences of probands were compared with those of historical controls and the published sequences (Acampora et al., '89; Hong et al., '95). Two polymorphisms were detected as heterozygous regions on sequencing gels. To read the sequence of the variant alleles, PCR product was subcloned to separate the maternal and paternal alleles by blunt-end cloning using the Zero Blunt PCR Cloning Kit (Invitrogen). Kanamycin-resistant clones were mini-prepped (Sambrook et al., '89), and inserts were excised with *Eco*RI. Sequencing reactions were then carried out on the plasmid DNA.

Deviations from the published sequence in the first exon of *HOXA1* and the first exon of *HOXB1* coincided with restriction sites and thus could be detected by digestion of PCR product with restriction enzymes. For each allele, at least 50 samples were examined by both sequencing and digestion to be certain that the two techniques yielded the same result, and the remaining subjects were tested by comparing the lengths of DNA fragments after PCR amplification and digestion. PCR products were digested overnight at 37°C with *Hph*I (New England Biolabs) for *HOXA1* or *Msp*I (Boehringer-Mannheim) for *HOXB1*. Digested products were electrophoresed on either a 10% nondenaturing polyacrylamide gel or a 4% Metaphor (FMC BioProducts) agarose gel and stained with ethidium bromide.

Study design and analysis

The frequency of allelic variants was assessed in probands, their relatives, and the two groups without an ASD diagnosis described above. The groups without an autism diagnosis, the convenience sample and the diversity panel, were tested to determine whether the variants were widespread in North America and other parts of the world. The probands and their relatives were evaluated by χ^2 for conformity to the three possible genotypes with Hardy-Weinberg proportions (two alleles, one degree of freedom, as described by Snustad and Simmons, '00). Nuclear families in the subject population ascertained through offspring with ASDs were used to determine whether (1) allele transmission from heterozygous parents to affected and unaffected offspring differed from Mendelian expectation, (2) the maternal and paternal gene transmission ratios differed from each other, and (3) there was an effect of sex of the offspring on gene transmission ratios. The results were tested by χ^2.

RESULTS

Mutation detection studies

In *HOXA1*, a single base substitution of guanine for adenine was found at base 218 (A218G) in a series of histidine repeats (Fig. 1A) when compared with the original published sequence (accession number U10421). The alternate sequence changes the codon for one histidine in a series of histidine repeats to an arginine at position 73. Since we identified this allele a PAC sequence containing the *HOXA1* gene (accession number AC004079) has been published; it also has the A218G substitution. A second deviation from the published sequence was detected in the first exon of *HOXA1* in an affected cousin of a proband and in her father. This variant allele included the same single base substitution as the A218G allele but, in addition,

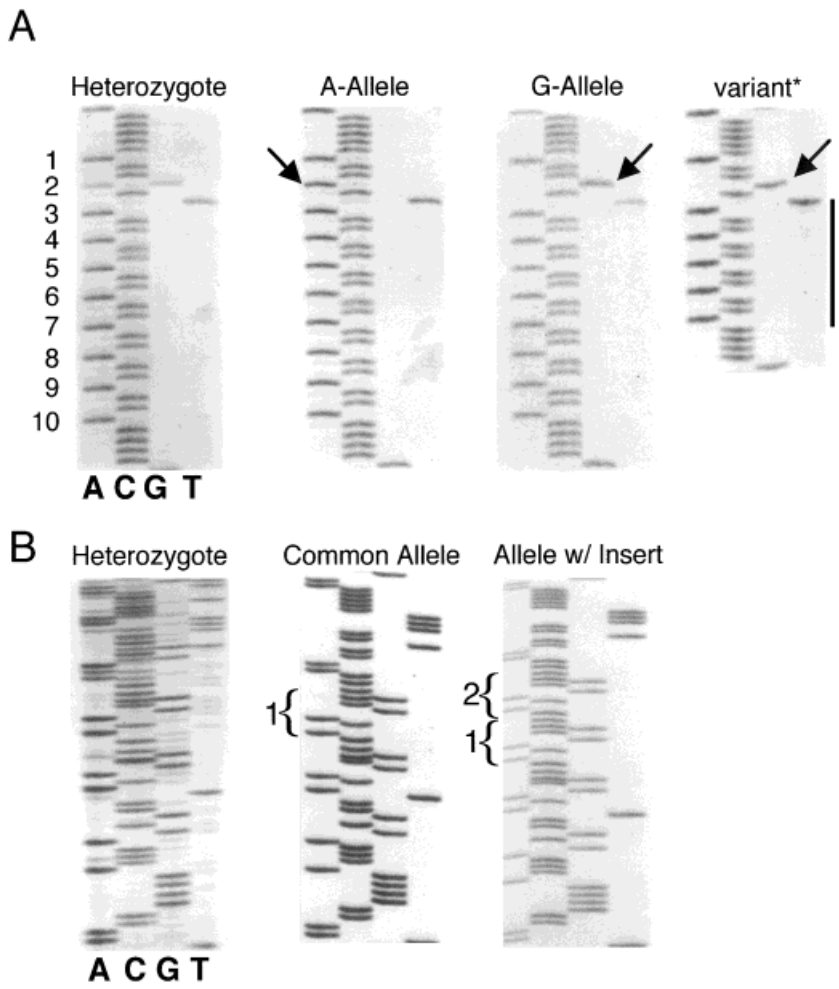

Fig. 1. Polymorphisms in exon 1 of *HOXA1* and *HOXB1*. **A:** Direct sequencing of *HOXA1* from polymerase chain reaction (PCR)-amplified genomic DNA and cloned PCR products. Arrows indicate a change at base 218 from adenine (A) to guanine (G), which alters the amino acid sequence from His to Arg. The first panel is a heterozygote, while panels 2 and 3 represent the A allele and G allele, respectively. Numbers on the left-hand side denote the histidine repeats. The last panel (variant*) is a polymorphic allele from an affected family member of a proband; in addition to the A-to-G substitution, this sequence has a deletion of three histidine codons in the region identified by the vertical line. One parent of this subject has the same variant allele of *HOXA1*. **B:** Direct sequencing of *HOXB1* from PCR-amplified genomic DNA and cloned PCR products. The first panel represents the heterozygous sequence. The common allele contains a sequence coding for Ser-Arg-His, as indicated by the numbered bracket (1{). In the other allele this sequence is repeated (2{).

three codons for histidine were absent from the series of histidine repeats (Fig. 1A).

A 9-base insertion after base 88 was identified in the first exon of *HOXB1* (accession number for published sequence X16666). The insertion codes for histidine-serine-alanine (Fig. 1B). In every case in which the insertion was present (but never in its absence), two additional sequence changes were observed. Thymidine was substituted for adenine at base 315 and adenine was substituted for guanine at base 456. The first substitution changes a codon for glutamine to one for histidine and the second has no effect.

Figure 2 shows the band patterns that resulted from digestion of PCR product from people of different genotypes. In every case, the variants at both loci appeared to be inherited from a parent, rather than occurring de novo.

Allele and genotype frequencies in diverse populations

The frequency of *HOXA1* genotypes in four subject populations is shown in Table 2 (A designates the published sequence [accession number U10421]; G designates the variant sequence, A218G). These data establish a high frequency polymorphism for the variant of *HOXA1* in individuals of European or African origin. No examples of the variant were found in 30 persons of Asian origin. The frequency of the G allele was 0.202 in the 57 probands and 0.203 in their 32 affected relatives. The frequency of the G allele in 134 unaffected relatives of subjects with an ASD was 0.164. The frequency of the G allele in the convenience population was 0.109.

In the population ascertained for an ASD proband, there was a significant deviation from Hardy-Weinberg proportions in *HOXA1* genotypes ($P = 0.005$; Table 3). The small increase in heterozygotes and larger decrease in homozygotes observed in this population occurred in both affected and unaffected family members. The absence of G/G homozygotes in the convenience population and also in the Diversity Panel subjects raises the possibility of a deficit of G/G homozygotes even in non-ASD populations.

The insertion variant of *HOXB1* was also established as a high-frequency polymorphism in individuals of European or African origin. In Table 2, + designates the allele with the published sequence and INS designates the allele with the insertion. Only one of 30 persons of Asian origin was a heterozygote. The frequency of the INS allele in the 57 probands was 0.254.

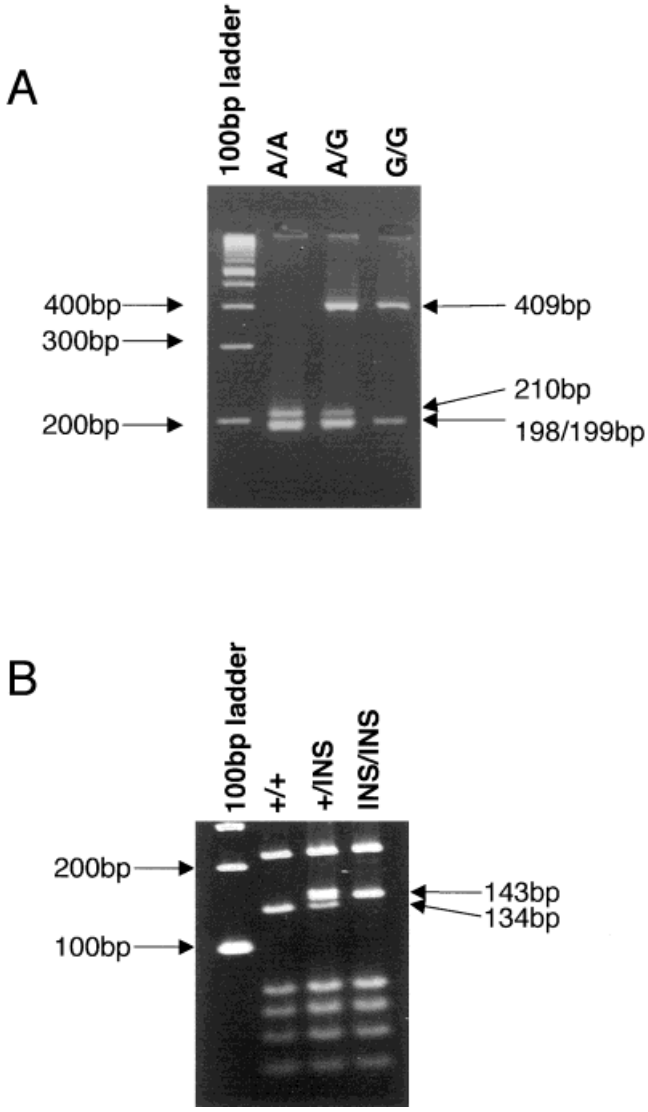

Fig. 2. Pattern of bands seen after digestion of *HOXA1* or *HOXB1* PCR product with restriction endonucleases. **A:** *HOXA1* PCR product was digested with the restriction endonuclease *Hph*I. The far left lane is a 100-bp ladder with 200-, 300-, and 400-bp fragments labeled on the left. *Lane 2* (A/A), represents the digestion pattern of product from a person homozygous for the A allele; *lane 3* (A/G), is from a heterozygous sample; *lane 4* (G/G), is from a sample homozygous for the G allele. Numbers along the right side indicate the size of the fragments: The 198/199 indicates the level where bands of either 198 bp or 199 bp, or both, appear on the gel. The 198 band is present in all three genotypes. In the A allele, digestion produces bands of 210, 199, and 198 bp, with the last two appearing as a single band. In the G allele, the presence of a G at base 218 results in the loss of a restriction site. Thus, a band of 409 bp is present and the 210- and 199-bp bands are absent. **B:** *HOXB1* PCR product was digested with the restriction endonuclease *Msp*I. The far left lane is a 100-bp ladder with 100- and 200-bp fragments labeled on the left. *Lane 2* (+/+), represents the digestion pattern of a PCR product of a sample from a homozygote with the published sequence; *lane 3* (+/INS), is from a heterozygous sample; *lane 4* (INS/INS) is from a sample homozygous for the 9-base insertion. Digestion of product with the common sequence gives a band at 134 bp. In the INS/INS homozygote, the insertion increases the size of the fragment by 9 bases to 143 bp.

The frequency was 0.307 among 31 affected relatives (one person typed for *HOXA1* was not typed for *HOXB1*). The frequency of the INS allele was 0.224 in 134 unaffected first- or second-degree relatives of probands and was 0.218 in the convenience population.

Assuming Hardy-Weinberg proportions for *HOXB1* genotypes among the 222 people ascertained through ASD probands, there was no statistically significant difference between the observed and expected number of genotypes. However, the increase in heterozygotes observed was nearly significant in the unaffected relatives of ASD probands ($P = 0.065$).

Gene transmission analyses

There were 68 affected (50 males and 18 females) and 40 unaffected offspring (13 males and 37 females) in the 50 nuclear families. *HOXA1* mating types of the 50 couples and genotypes of the 66 affected and 16 unaffected offspring who were typed are summarized in Table 4. Given the evidence for a slightly increased frequency of A/G heterozygotes and a substantially decreased frequency of G/G homozygotes in the ASD population, we compared the number of heterozygotes and homozygotes among the 48 offspring of segregating matings with expectations based on Mendelian segregation. Among the 40 affected offspring of 30 segregating matings, there were 28 heterozygotes and 12 homozygotes as compared with equal numbers of each expected ($\chi^2_{1df} = 6.4$, $P = 0.011$). Among the eight unaffected offspring of segregating matings, the ratio was four heterozygotes to four homozygotes, the same as expectation.

Table 5 shows the same families broken down by mating types and sex of offspring. There was no significant difference between the number of *HOXA1* heterozygous mothers and heterozygous fathers, and the frequency of each mating type did not differ significantly from random expectation based on genotype frequencies in mothers and fathers. Although it appears from Table 5 that *HOXA1* A/G heterozygous fathers produced a higher proportion of affected offspring (20/21) than A/A fathers (46/61), there was little difference in the proportions of affected offspring when the 26 offspring who were not typed for *HOXA1* were included (20/31 from A/G fathers as compared with 48/77 from A/A fathers). The proportion of affected offspring from A/G mothers was 26/46 and from A/A mothers was 42/62.

Inspection of Table 5 reveals two unusual features of the distribution of *HOXA1* genotypes in families ascertained for ASDs. First, much of the increase in the number of heterozygotes in the affected offspring occurred in matings of A/G mothers by A/A fathers: 16 heterozygotes and 4 homozygotes, compared with equal numbers expected ($\chi^2_{1df} = 7.2$, $P = 0.007$). Second, there appears to be an effect of sex on the distribution of *HOXA1* genotypes in the offspring of the 30 segregating matings. All nine affected females were heterozygotes, compared with equal numbers of heterozygotes and homozygotes expected ($\chi^2_{1df} = 9.0$, $P = 0.003$). The heterozygote excess in females was even

TABLE 3. Number of persons with A/G and +/INS genotypes (expected numbers based on hardy-weinberg proportions)

		HOXA1 genotypes		
ASD probands and relatives				
	A/A	A/G	G/G	
Observed	144	78	1	
Expected	150.31	65.54	7.15	$\chi^2 = 7.92$
Affected ASD family members				
	A/A	A/G	G/G	
Observed	54	34	1	
Expected	56.68	28.69	3.63	$\chi^2 = 3.01$
Unaffected ASD family members				
	A/A	A/G	G/G	
Observed	90	44	0	
Expected	93.65	36.74	3.61	$\chi^2 = 5.18$
		HOXB1 genotypes		
ASD probands and relatives				
	+/+	+/INS	INS/INS	
Observed	122	92	8	
Expected	127.22	83.67	13.11	$\chi^2 = 3.04$
Affected ASD family members				
	+/+	+/INS	INS/INS	
Observed	45	38	5	
Expected	46.51	34.93	6.56	$\chi^2 = 0.69$
Unaffected ASD family members				
	+/+	+/INS	INS/INS	
Observed	77	54	3	
Expected	80.69	46.59	6.72	$\chi^2 = 3.41$

ASD, autism spectrum disorder.

TABLE 4. Autism spectrum disorders: *HOXA1* genotypes of 66 affected offspring and 16 unaffected offspring from 50 parent pairs

		No. of affected offspring with genotype			No. of unaffected offspring with genotype		
Mating type	No. of matings	A/A	A/G	G/G	A/A	A/G	G/G
Both parents A/A	20	26	0	0	8	0	0
One or two parents A/G	30	11	28	1	4	4	0

more striking if the five unaffected female offspring were included; i.e., among all female offspring, the A/G heterozygote:homozygote ratio was 13:1, compared with equal numbers expected ($\chi^2_{1df} = 10.29$, $P = 0.0013$). In contrast, among all the male offspring of these same matings, the heterozygote-to-homozygote ratio was 19:18.

HOXB1 mating types and offspring genotypes are summarized in Table 6 and detailed in Table 7. There was no significant difference between the number of heterozygous mothers and fathers, and mating types did not differ from random expectations based on genotype frequencies in mother and fathers. There were 30 affected male, 9 affected female, 3 unaffected male, and 9 unaffected female offspring from 30 segregating matings. Among the 39 affected offspring of segregating matings there were 22 heterozygotes and 17 homozygotes, and among unaffected offspring, 4 heterozygotes and 8 homozygotes. Neither of these proportions was significantly different from the equal numbers expected. The transmission ratios of the + and INS alleles from segregating matings to affected offspring were 22 + and 32 INS. To unaffected offspring they were 9 + and 6 INS. Again, neither result was significantly different from the equal numbers expected. However, it is noteworthy that by either method of evaluation the proportions were in the opposite direction in affected as compared with unaffected offspring, with a trend toward increased heterozygosity occurring only in those who were affected.

TABLE 5. Autism spectrum disorders: *HOXA1* mating types of 50 parent pairs and genotypes of 66 affected offspring and 16 unaffected offspring

Mating type		No. of matings[a]	No. of affected offspring with genotype						No. of unaffected offspring with genotype					
			Males			Females			Males			Females		
Mother	Father		A/A	A/G	G/G	A/A	A/G	G/G	A/A	A/G	G/G	A/A	A/G	G/G
A/A	A/A	20 (21.1)	18	0	0	8	0	0	1	0	0	7	0	0
A/A	A/G	11 (9.9)	5	6	0	0	3	0	1	0	0	0	0	0
A/G	A/A	14 (12.9)	4	12	0	0	4	0	2	0	0	1	4	0
A/G	A/G	5 (6.1)	2	1	1	0	2	0	0	0	0	0	0	0

[a] Expected number of mating types, shown in parentheses, were determined from the observed frequency of each genotype in mothers and fathers.

TABLE 6. Autism spectrum disorders: *HOXB1* genotypes of 65 affected offspring and 16 unaffected offspring from 50 parent pairs

Mating type	No. of matings	No. of affected offspring with genotype			No. of unaffected offspring with genotype		
		+/+	+/INS	INS/INS	+/+	+/INS	INS/INS
Both parents +/+	18	23	0	0	4	0	0
One or two parents +/INS	30	12	22	5	7	4	1
One parent +/+, one INS/INS	2	0	3	0	0	0	0

TABLE 7. Autism spectrum disorders: *HOXB1* mating types of 50 parent pairs and genotypes of 65 affected offspring and 16 unaffected offspring

Mating type		No. of matings[a]	No. of affected offspring with genotype						No. of unaffected offspring with genotype					
			Males			Females			Males			Females		
Mother	Father		+/+	+/INS	INS/INS	+/+	+/INS	INS/INS	+/+	+/INS	INS/INS	+/+	+/INS	INS/INS
+/+	+/+	18 (16.8)	16	0	0	7	0	0	1	0	0	3	0	0
+/+	+/INS	9 (11.0)	3	5	0	1	1	0	0	1	0	2	1	0
+/INS	+/+	11 (12.2)	4	7	0	2	1	0	1	1	0	2	1	0
+/INS	+/INS	10 (8.0)	2	6	3	0	2	2	0	0	0	2	0	1
+/+	INS/INS	2 (1.2)	0	2	0	0	1	0	0	0	0	0	0	0

[a] Expected number of mating types, shown in parentheses, were determined from the observed frequency of each genotype in mothers and fathers.

Characteristics of the phenotypes

The basic phenotypic features were evenly distributed over cases with different *HOXA1* and *HOXB1* genotypes. In particular, neither the diagnostic categories of the autism spectrum, degree of cognitive impairment, nor neurologic and morphologic symptoms appeared to be correlated with genotype (Table 1). Some distinction might be demonstrable in larger samples, but the present data suggest that neither allele is associated with any particular subset of cases within the autism spectrum.

Several phenotypic characteristics not ordinarily associated with autism were observed among the probands. There was one case of Marfan syndrome (A/G, +/INS). This proband and his brother bring the number of cases reported to be comorbid for Marfan syndrome and ASDs to six (Tantam et al., '90; Chudley et al., '98). There was one proband with syndactyly (A/G, +/INS), one with Tourette syndrome (A/A, +/+), and another case with a proximal limb anomaly, unilateral anotia, and scoliosis who was possibly exposed to rubella in utero (A/A, +/+). Another case with probable intrauterine infection had facial diplegia, hypertelorism, and ptosis, and was blind (A/G, +/+). Two probands had brachydactyly type four of the thumb (A/A, +/+; A/A, +/INS) and two had clinodactyly of the fifth digit (A/A, +/+; A/A, +/INS). Two of the probands had been exposed in utero to chemical teratogens associated with autism—one to valproic acid and one to ethanol. Both of these cases were A/G for *HOXA1* and +/+ for *HOXB1*.

The neuroanatomy of the brain of a person with autism was one of the sources of evidence that suggested *HOXA1* and *HOXB1* as candidate genes in ASDs (Rodier et al., '96). Genomic DNA extracted from the brain tissue was tested for the alternate alleles of each gene. That person was A/A for *HOXA1* and was +/INS for *HOXB1*.

DISCUSSION

In four large genome screen studies of autism published in the last two years, there is no region that consistently shows significantly increased haplotype sharing in affected sib pairs. The International Molecular Genetic Study of Autism Consortium ('98) found the greatest evidence for susceptibility on the long arm

of chromosome seven (7q31), in a region previously linked to a rare speech and language disorder (Fisher et al., '98). The French international study (Philippe et al., '99) found a minor peak (MLS = 0.83) in the same region, the Stanford group (Risch et al., '99) found no evidence of genetic susceptibility, and the Collaborative Linkage Study of Autism ('99) found a LOD score of 2.2. The last study found its highest LOD score (3.0) on chromosome 13, where none of the others had found any evidence of linkage. Cases of autism with a translocation on chromosome fifteen have been reported (e.g., Cook et al., '97). Although the translocation is not characteristic of many cases, polymorphisms of genes in the region of the chromosomal defect could be important etiological factors (Cook et al., '98), but only the French study found a MLS score as high as 1.0 in this region. The study from Stanford found only four regions with MLS scores of 1.0 or more. One of these was on the short arm of chromosome 7 in the region of the *HOXA* cluster (Risch et al., '99). However, there was no evidence of linkage in this region in the other studies. On the basis of the small size of LOD scores generated from these genome screens, Risch and colleagues ('99) have suggested that more than fifteen loci are likely to be involved in susceptibility to ASDs.

In this article, we have reported a base substitution variant, A218G, at the *HOXA1* locus and an insertion variant at the *HOXB1* locus. In the case of the *HOXA1* locus, the string of histidine repeats in the first exon of *HOXA1* disrupted by the substitution is believed to be the site at which the *HOXA1* protein binds to other proteins, just as the homeobox in the second exon allows binding to DNA. It is one of the most highly conserved regions of the gene, varying only in the length of the histidine series: 9 in the rat, 10 in the human, and 11 in the mouse. Thus, it may well be that the G allele, in which an arginine is substituted for one of the histidines, has functional characteristics different from the A allele. The rarity of G/G homozygotes in comparison with expectation supports the hypothesis that the G/G phenotype confers some disadvantage, providing additional evidence that the A and G alleles are functionally different.

The region of the insertion in *HOXB1* differs between the human and the mouse: the region of the allele that is repeated in some humans codes for histidine-serine-alanine, while it codes for proline-serine-alanine in the mouse. Although an insertion might well be functionally disruptive, the base sequence in this region may not be critical to function. Both variants are present in high frequency in persons of European or African ancestry. Given this high frequency and a possible functional effect, both variants are candidates for significantly influencing the development of the hindbrain and associated structures in the population as a whole. By the arguments presented in the Introduction, one of the effects of certain genic combinations at these loci might include susceptibility to ASDs.

Hox genes modulate other genes in early embryogenesis and are highly conserved across species. Previously, genetic variation at either *Hoxa1* or *Hoxb1* had not been described in any mammalian species. In mice with null mutations of *Hoxa1*, homozygosity is lethal soon after birth (Carpenter et al., '93; Mark et al., '93). Homozygosity for a null mutation of *Hoxb1*, on the other hand, has less effect on viability (Studer et al., '96). The present findings indicate that homozygosity for an alternate allele of *HOXA1* may decrease viability in humans, as homozygosity for null mutations of the gene does in the mouse, while there is no evidence of reduction of the frequency of homozygotes with the INS/INS genotype of *HOXB1*.

Examination of heterozygotes in the transgenic mouse models of *Hoxa1* and *Hoxb1* has not indicated any obvious phenotypic effects. There is evidence that humans are more affected than mice are by a single copy of some polymorphisms of early developmental genes. For example, deviation in function of the gene *sonic hedgehog* causes variable midline deficits including holoprosencephaly in humans heterozygous for an alternate allele, while mice are affected only if they are homozygous for the null mutation (Chiang et al., '96; Roessler et al., '96).

The *HOXA1* gene transmission analyses were performed using individuals who were included in the comparisons with Hardy-Weinberg proportions. Thus, the family-based analyses are not completely independent in a statistical sense from the population analyses, but they do have different characteristics. The gene transmission analyses have the disadvantage of being applicable only to heterozygous parents—a subset of the total population—but have the advantage of requiring no assumptions about population structure. Tests of the ratio of heterozygous to homozygous offspring showed a statistically significant deviation from Mendelian expectations. The deviation was observed in 40 affected offspring, and not in 8 unaffected offspring. The increased heterozygosity in affected offspring supports the hypothesis that either the *HOXA1* locus, or a locus in linkage disequilibrium with *HOXA1*, is related to ASDs. This conclusion must remain tentative until it can be determined whether the A:G segregation distortion observed in affected offspring is absent in a larger sample of unaffected offspring in the same families, and also in families not ascertained for ASDs.

The sex difference in diagnosis of autism and Asperger syndrome has long puzzled investigators. The most disabled, mentally retarded cases of ASDs occur at a 1:1 sex ratio, while higher-functioning individuals exhibit a 10:1 male-to-female ratio (Gillberg, '89). These observations fit with a notion that females have decreased penetrance of the autism phenotype rather than reduced viability. The most compelling explanation of the sex difference proposed thus far is based on data from girls with Turner syndrome, who have only one normal X-chromosome. The phenotype of the syndrome is variable, with some individuals exhibiting few cognitive disabilities, and others exhibiting mental retardation and social deficits that sometimes result in a diagnosis of autism. Skuse et al., ('97) determined that the cases with cognitive deficits are those who have maternal X-chromosomes, while those with more normal behavior have paternal X-chromo-

somes. The authors have proposed that a genetic factor that enhances the development of social behaviors exists on the X-chromosome; it is imprinted on the maternal X-chromosome and functional only on the paternal X-chromosome. Such a factor would explain the findings in Turner syndrome, the increased expression of social behaviors in normal females compared with normal males, and the apparent resistance of females to susceptibility to autism. In a subsequent report (Creswell and Skuse, '99), none of 65 Turner syndrome females with complete paternal X-chromosomes had a diagnosis of autism, while 10 of 156 with complete maternal X-chromosomes had the diagnosis. The putative protective factor fits well with many facts about autism (Skuse, '00). It predicts that females diagnosed with autism have more or stronger risk factors than males with the same diagnosis. If this is true, it would not be surprising if some potent risk factors are present in a higher proportion of female cases than male cases of autism.

In this context, the gender effect on *HOXA1* genotypes has special interest. There were only 17 affected female offspring in our study and only 9 from A/G heterozygous parents. All 9 were A/G heterozygotes. Clearly, A/A homozygous females can develop autism, and in a larger sample there are likely to be some A/A affected female offspring from A/G parents. Nevertheless, the gene transmission results in this initial study suggest that the *HOXA1* effect may be more apparent in females than in males. Indeed, the data from the total population are consistent with this notion: the ratios of A/G to A/A genotypes in affected individuals are 11:10 in females and 23:44 in males.

The idea that the parental source of alleles is important in autism has been suggested by family studies (Szatmari et al., '95), observations of the behavioral phenotype associated with inherited chromosomal anomalies in families (Cook et al., '97), and studies of identity-by-descent sharing (Ashley-Koch et al., '99). The region of the human *HOXA* cluster is homologous with mouse chromosome 6 B–C, where maternal imprinting has been detected (Searle et al., '89), but the region has not been studied in detail in either species. It has often been suggested that paternal imprinting is temporarily active at many loci in embryonic tissue (Strachan and Read, '99). The segregation data presented in Table 4 are insufficient to address this question, but a larger proportion of affected offspring with a maternal G allele (16 out of 20) as opposed to a paternal G allele (9 out of 14) is not inconsistent with an imprinting effect.

Analyses of *HOXB1* genotypes similar to the analyses of *HOXA1* genotypes did not result in any individual statistically significant results. Nevertheless, similar deviations from random expectations were observed: a small increase in heterozygosity over expectations based on Hardy-Weinberg proportions in the population analysis, and greater INS than + allele transmission to affected offspring in the segregation analysis. On the assumption that several loci contribute to the susceptibility to ASDs, we hypothesized that the effect at a single locus might be most obvious if the contributions of potential susceptibility alleles at other loci were taken into consideration. To test this, we compared the *HOXA1* heterozygote-to-homozygote ratio in affected individuals who were female (less frequently affected and possibly less susceptible than males) and who did not have a *HOXB1* INS allele with the *HOXA1* heterozygote-to-homozygote ratio in unaffected individuals with an INS allele. We hypothesized that unaffected subjects with an INS allele, especially if male, would be the least likely to be *HOXA1* heterozygotes. The A/A-to-A/G ratio in the INS negative affected females was 3:9, as compared with an A/A-to-A/G ratio in unaffected *HOXB1* INS-positive males of 16:7 (contingency $\chi^2_{1df} = 6.30$, $P = 0.012$) and in unaffected *HOXB1* INS-positive females of 24:10 (contingency $\chi^2_{1df} = 7.58$, $P = 0.0059$). More data on the A/A-to-A/G ratio in INS negative affected females are required, but these initial results point to possible interaction between gender and genotypes of *HOXA1* and *HOXB1* in ASD susceptibility. Such complex genetic interactions may explain the failure to find significant evidence of a single locus effect in the studies of genome screening in affected sib pairs.

This sample of probands with autism spectrum disorders, like others reported previously, exhibited mild phenotypic features (e.g., esotropia, mild external ear malformations, facial hypotonia) that one would expect to occur in people with altered function of either *HOXA1* or *HOXB1*. These features were distributed evenly across the *HOXA1* and *HOXB1* genotypes studied here. Interestingly, several of the unexpected phenotypic features observed in the sample are related to expression of other genes in the *Hox* cascade. For example, proximal and distal limb anomalies are seen in both genetic and teratologic disturbances of the *Hox* genes (Means and Gudas, '95). Because expression of *HOXA1* and *HOXB1* influence the expression of the subsequently activated *HOX* genes (Simeone et al., '90; Hong et al., '95), it is possible that these physical anomalies are generated in the probands by alteration of the function of other genes secondary to changes in the function of *HOXA1* or *HOXB1*.

What makes the present results especially interesting is not just the significant distortion of *HOXA1* genotype frequencies in families ascertained for ASDs, or the significant deviation from expected rates of allele transmission, but the fact that the biological plausibility of a role for this locus in autism is supported by so many lines of converging evidence. Examples include the observation of many of the same morphologic and neurologic anomalies in thalidomide- and valproate-induced autism, idiopathic autism, and mice with null mutations of *Hoxa1*; the coincidence of the embryological stage when teratogens induce autism with the stage when *HOXA1* is expressed; and the peak at this locus in a genome screen for autism.

Three recent studies of rare genetic syndromes provide yet another type of evidence regarding the association of developmental alteration of the brain stem and autism. A prospective study of cases of Möbius syndrome in Sweden indicates that at least 24% have

autism along with the dysfunctions of the facial and abducens nerves that define the syndrome (Miller et al., '98). Joubert syndrome is characterized by a severe midline defect of the brain stem and cerebellar vermis, accompanied by dysfunctions of cranial nerves (Joubert et al., '69; Holroyd et al., '91). When eleven cases ascertained for Joubert syndrome were tested for ASDs, four met the diagnostic criteria (Ozonoff et al., '99). Duane syndrome (failure of innervation of the lateral rectus by the abducens nerve, with reinnervation by the oculomotor) can be induced by teratogens and was present in three of the four thalidomide-induced cases of autism reported by Strömland and colleagues ('94). A locus for this syndrome has just been identified, and it coincides with the *HOXD* cluster at chromosome 22q31(Appukuttan et al., '99). This makes *HOXD1*, the third paralog of *HOXA1,* an excellent candidate gene for Duane syndrome.

There is a growing body of literature supporting the hypothesis that early injury to the developing brain stem increases the risk for autism spectrum disorders. The present findings add evidence that loci critical to brain stem development have a role in susceptibility to ASDs.

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